Persistence of botulinum neurotoxin action in cultured spinal cord cells[1,2]

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Abstract Primary dissociated fetal mouse spinal cord cultures were used to study the mechanisms underlying the differences in persistence of botulinum neurotoxin A (BoNT/A) and botulinum neurotoxin/E (BoNT/E) activities. Spinal cord cultures were exposed to BoNT/A (0.4 pM) for 2–3 days, which converted approximately half of the SNAP-25 to an altered form lacking the final nine C-terminal residues. The distribution of toxin-damaged to control SNAP-25 remained relatively unchanged for up to 80 days thereafter. Application of a high concentration of BoNT/E (250 pM) either 25 or 60 days following initial intoxication with BoNT/A converted both normal and BoNT/A-truncated SNAP-25 into a single population lacking the final 26 C-terminal residues. Excess BoNT/E was removed by washout, and recovery of intact SNAP-25 was monitored by Western blot analysis. The BoNT/E-truncated species gradually diminished during the ensuing 18 days, accompanied by the reappearance of both normal and BoNT/A-truncated SNAP-25. Return of BoNT/A-truncated SNAP-25 was observed in spite of the absence of BoNT/A in the culture medium during all but the first 3 days of exposure. These results indicate that proteolytic activity associated with the BoNT/A light chain persists inside cells for >11 weeks, while recovery from BoNT/E is complete in <3 weeks. This longer duration of enzymatic activity appears to account for the persistence of serotype A action.

Key words: Botulinum neurotoxin; Synaptosomal-associated protein of 25 kDa (SNAP-25); Spinal cord culture

1. Introduction

The botulinum neurotoxins (BoNTs) comprise a family of highly lethal protein toxins that act by selectively inhibiting the release of acetylcholine at the neuromuscular junction and autonomic ganglia [1–3]. The toxins are produced by strains of the anaerobic bacterium *Clostridium botulinum* and are responsible for foodborne, infant and wound botulism [1–3]. Seven immunologically distinct serotypes of BoNT are known and are designated in the order of their discovery as A–G [1,2]. In their active form, these clostridial neurotoxins are dichain proteins associated by a single disulfide bond [4]. The larger subunit (heavy chain, ~100 kDa) mediates toxin binding and internalization; the smaller subunit (light chain, ~50 kDa) possesses zinc metalloprotease activity and cleaves one of three proteins involved in the docking and release of synaptic vesicles: synaptobrevin, syntaxin and SNAP-25 [5–7]. BoNT/A and E cleave SNAP-25 near its C-terminus, removing nine and 26 residues, respectively [8].

Although BoNT/A and E both target SNAP-25, the two serotypes differ markedly in duration of action. Intoxication by serotype E is relatively short-lived, whereas paralysis caused by serotype A is persistent, lasting for up to 6 months [9,10]. The persistence of BoNT/A has led to its use in a variety of muscle tone and movement disorders [11].

The basis for differences in paralysis times is not well understood. It may reflect differences in the stability of the respective light chains in the nerve terminal or differences in the inherent toxicity of SNAP-25 cleavage products [12]. Consistent with the latter proposal, Ferrier-Monteil et al. [13] found that a synthetic peptide identical to the 26-mer BoNT/E cleavage product inhibits transmitter release in bovine chromaffin cells and in *Aplysia* buccal ganglia driver cells. This peptide appears to act by forming a non-productive fusion complex with the synaptic vesicle protein synaptobrevin and the active zone protein syntaxin [13].

Evidence in support of the former proposal has been obtained by sequential local injections of BoNT/A and E in rat extensor digitorum longus muscle [14]. Injection of BoNT/A before or after BoNT/E led to paralysis times that were controlled by BoNT/A rather than by BoNT/E. These data suggest that the duration of toxicity is determined by the lifetime of active BoNT/A.

A more direct test of the factors underlying toxin persistence would require monitoring the lifetime of BoNT or of the state of SNAP-25 in the nerve terminal. Detection of BoNT is inherently difficult due to the paucity of intracellular toxin [15], whereas monitoring of SNAP-25 is impractical in skeletal muscle since the nerve terminal comprises only a small proportion of the total muscle protein. To overcome these limitations, we used primary fetal spinal cord cultures to study the mechanisms underlying differences in persistence of BoNT/A and E. The results indicate that proteolytic activity associated with the BoNT/A light chain persists inside cells for over

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[2] In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996.

Abbreviations: BoNT/A, botulinum neurotoxin A; BoNT/E, botulinum neurotoxin E; SNAP-25, synaptosomal-associated protein of 25 kDa; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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11 weeks while recovery from BoNT/E is complete within 3 weeks. This longer duration of enzymatic activity appears to account for the persistence of serotype A action.

2. Materials and methods

2.1. Materials

Monoclonal antibodies to SNAP-25 and syntaxin were obtained from Sternberger Monoclonals, Inc. (Baltimore, MD, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. The polyclonal anti-mouse antibody conjugated to alkaline phosphatase was also purchased from Sigma. All electrophoresis reagents were from Bio-Rad (Hercules, CA, USA). Polyvinylidene difluoride membrane (PVDF) was obtained from either BioRad (0.2 μm) or Sigma (0.4 μm). Non-fat dry milk was from Nestle Food Co. (Glendale, CA, USA).

2.2. Animals

Timed pregnant C57BL/6J mice were obtained from the Frederick Cancer Research and Development Center, Frederick, MD, USA. Animals were rendered unconscious in a CO_2 atmosphere and decapitated. Embryos were surgically removed from the amniotic sac, decapitated and the spinal cords were isolated for preparation of dispersed cell cultures.

2.3. Cell culture

Spinal cord cell cultures were prepared as described [16,17]. Briefly, spinal cords were removed from fetal mice at gestation day 13, dissociated with trypsin and plated on Vitrogen coated dishes (Collagen Corp., Palo Alto, CA, USA) at a density of 10^6 cells/dish. Cultures were maintained for 3 weeks at 37°C in an atmosphere of 90% air/10% CO_2 before addition of toxins. Cultures were grown in Eagle's minimum essential medium (formula 82-0234AJ; Life Technologies Inc., Bethesda, MD, USA) supplemented with 5% heat-inactivated horse serum and a mixture of complex factors [18].

2.4. Preparation of protein extracts, gel electrophoresis and Western blot analysis

2.4.1. Preparation of total protein. Protein was prepared by dissolving cells in a 1% SDS solution. The slurry was transferred to 1.5-ml microfuge tubes, incubated in a 95°C water bath for 10 min to inactivate proteases and stored at −30°C. Protein concentration was determined by a modification of the bicinchoninic method (Pierce Chemical Co., Rockford, IL, USA). Samples were mixed with equal volumes of SDS-reducing buffer (2% SDS, 5% β-mercaptoethanol, 55 mM Tris-Cl, pH 6.8, 10% glycerol and 0.05% bromophenol blue) and heated at 95°C for 5 min immediately prior to separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4.2. Gel electrophoresis and Western blotting. Equal quantities of protein were loaded onto 16.5% acrylamide gels by the method of Laemmli [19]. Proteins were separated using 0.1 M Tris-Tricine as run buffer (pH 8.3) [20] then transferred to PVDF membrane using 192 mM glycine, 25 mM Tris, pH 8.3 and 7% methanol.

The PVDF membrane was treated with Tris-buffered saline blocking buffer (TBS blocker: 0.5 M NaCl, 25 mM Tris, pH 7.5 containing 4% non-fat dry milk) for 1 h. Primary antibodies in blocking buffer were incubated with the membrane at 30°C using gentle agitation for 90 min followed by two sequential 10-min washes with TTBS (TBS containing 0.05% Triton X-100) at room temperature. A secondary antibody conjugated to alkaline phosphatase was similarly applied to the membrane, followed by two sequential washes with TTBS, one wash with TBS and a 1-min rinse with deionized water before visualizing the bound alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium solution (Sigma). Antibodies were diluted 1:1000 in blocking buffer prior to use.

2.5. Botulinum neurotoxin preparation

Preparations of BoNT/A and E toxin complex were from Wako Chemicals (Richmond, VA, USA) with reported activities of 2.0×10^7 LD_{50} per mg. BoNT/E (1 mg/ml) was activated (nicked) by incubating for 30 min at 37°C with 0.3 mg/ml trypsin (type XI, bovine pancreas) in 30 mM HEPES, pH 6.75 [21]. Soybean trypsin inhibitor type I-S (0.5 mg/ml) was added in the same buffer and incubated at room temperature for 15 min to inhibit further trypsinization. Toxin was aliquoted and stored at −30°C. Stock solutions of BoNT/A (1 mg/ml) were diluted to 100 nM in a solution consisting of 200 mM NaCl and 50 mM Na acetate (pH 6.0), aliquoted and stored at −30°C. Each experiment utilized a new aliquot of toxin to ensure uniform activity. Molar concentrations stated in the text where based upon masses of 500 and 300 kDa for BoNT/A and E, respectively. Toxins were dissolved in supplemented MEM, and cultures were incubated with BoNT for 18–72 h, as appropriate. BoNT-containing media was removed by aspiration; cells were rinsed with toxin-free MEM then fed with 2 ml of control supplemented MEM. Feeding then resumed to half-changes of supplemented MEM twice weekly.

2.6. Data analysis

Scanned images of Western blots were produced and stored utilizing Photoshop (Adobe Systems, Inc., Mountain View, CA, USA). When indicated, images were digitally analyzed using ONE-Dscan gel analysis software (Scanalytics, Inc., Fairfax, VA, USA). Densitometric values were plotted using non-linear regression analysis (GraphPad Prism 3.0, San Diego, CA, USA). Error bars represent one standard deviation of triplicate determinations.

3. Results

3.1. Specific proteolysis by BoNT/A and E

Fig. 1 demonstrates proteolysis of SNAP-25 by BoNT/A and E in cultured mouse spinal cord cells by Western blot analysis. Control cells contained abundant SNAP-25 (lane 1), which was contributed almost exclusively by the neuronal component of the cultures; glia have been reported to be devoid of SNAP-25 [22,23]. Incubation of cultures with 0.4 pM BoNT/A for 48 h led to cleavage of approximately 50% of the total neural SNAP-25 (lane 2). BoNT/A-truncated SNAP-25 has an apparent molecular weight of 24 kDa, due to loss of a 9-mer fragment from cleavage between Q197 and R198 (Table 1) [24]. Incubation of spinal cord cells with 36 pM BoNT/E produced an approximately equal degree of proteolysis of SNAP-25 (lane 3). The resulting BoNT/E-truncated SNAP-25 was smaller (22 kDa), since a 26-mer fragment was removed by specific cleavage between amino acids R180 and I181 (Table 1) [25]. The SNAP-25 antibody recognizes the N-terminal region, and therefore the nine and 26 C-terminal cleavage products could not be resolved in this study.

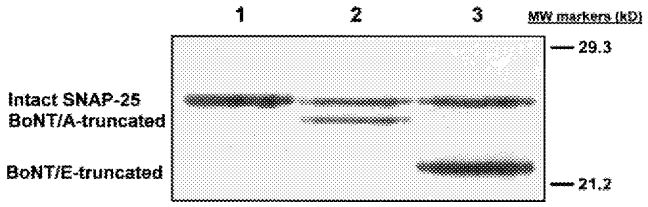

Fig. 1. Specific proteolysis of SNAP-25 after exposure of mouse spinal cord cell cultures to BoNT/A or E. Protein samples were prepared as described in Section 2 and subjected to SDS-PAGE under reducing conditions. The sample in lane 1 shows a band at approximately 25 kDa corresponding to SNAP-25 from untreated cells. Cultures treated for 48 h with either 0.4 pM BoNT/A (lane 2) or 36 pM BoNT/E (lane 3) exhibit approximately 50% proteolysis of SNAP-25. Production of the 24- and 22-kDa fragments (Table 1) is indicative of appropriate BoNT activity. The Western blot was screened using a monoclonal antibody that recognizes the same epitope on all three forms of SNAP-25. The smaller cleavage products of BoNT/A and E action do not appear in this or subsequent blots since the antibodies do not recognize the C-terminal region of SNAP-25.

3.2. Recovery of SNAP-25 from BoNT/E-mediated proteolysis

To determine the recovery of SNAP-25 following BoNT/E-mediated proteolysis, spinal cord neurons were exposed to BoNT/E, washed with control medium to remove excess toxin and monitored for restoration of intact SNAP-25 during 18 days in culture. The results of a representative experiment are shown in Fig. 2A. Under control conditions, a single 25 kDa band was apparent for SNAP-25 (lane 1). Following exposure to 116 pM BoNT/E for 18 h, nearly complete cleavage of SNAP-25 was evident (lane 2). Upon removal of excess toxin, a time-dependent recovery of intact SNAP-25 and disappearance of BoNT/E-altered SNAP-25 were observed (lanes 3–7). Recovery of intact SNAP-25 was rapid, such that nearly 20% of control density returned within 1 day and 90% recovery was evident 7 days after cells were returned to control medium (half-time = 5.8 days) (Fig. 2B). The disappearance of BoNT/E-truncated SNAP-25 occurred at a similar rate; approximately half of this toxin-altered protein disappeared within 4.6 days and little was apparent after 18 days of BoNT/E exposure.

3.3. Recovery of SNAP-25 from BoNT/A-mediated proteolysis

Unlike the relatively rapid restoration of intact SNAP-25 following BoNT/E exposure, little recovery of SNAP-25 was observed subsequent to BoNT/A intoxication. For evaluating BoNT/A, a low toxin concentration was selected to ensure that the slow recovery could not be attributed to excess intracellular toxin. As evident from the Western blots (Fig. 3A) and densitometric analysis (Fig. 3B), exposure of cultures to 0.4 pM BoNT/A resulted in approximately 40% cleavage of SNAP-25 after 3 days; little recovery of intact SNAP-25 or disappearance of BoNT/A-altered SNAP-25 was observed over an 80-day period (Fig. 3B). The persistence of serotype A action in spinal cord cultures was even more pronounced than that observed in rat EDL muscle after a paralytic dose of BoNT/A [9,10,14]. In the latter preparation, 42% of the control twitch tension returned 30 days after a local i.m. injection of BoNT/A [14]. These results suggest that recovery of muscle tension after BoNT/A is due largely to formation of new synapses rather than to restoration of function in BoNT/A-intoxicated nerve terminals [26].

3.4. Recovery of SNAP-25 from sequential application of BoNT/A and BoNT/E

The continued presence of BoNT/A-truncated SNAP-25 observed in Fig. 3 may reflect a static intracellular condition where SNAP-25 synthesis and degradation are both inhibited, or alternatively, it demonstrates persistent BoNT/A enzymatic activity. To gain insight into underlying the differences in persistence of BoNT/A and E on cleavage of SNAP-25, experiments were performed in which cells were incubated sequentially in BoNT/A and E, and SNAP-25 recovery rates were monitored at 43 and 78 days after addition of BoNT/A. Fig. 4 shows the results of two such studies. Addition of 0.4 pM BoNT/A for 72 h resulted in cleavage of ~50% of SNAP-25, which was undiminished 25 days later (Fig. 4, lane 1). Excess BoNT/A was removed by washout with control medium and cultures were maintained as before. Twenty-five days after exposure to BoNT/A, cells were incubated in 250 pM BoNT/E (Fig. 4A, lane 2); consequently, both intact and 24 kDa BoNT/A-truncated SNAP-25 were converted to the 22 kDa BoNT/E-truncated form. The BoNT/E-truncated SNAP-25 diminished over the next 13 days (Fig. 4A, lanes 3–5) and was only detectable in trace amounts by day 18 (Fig. 4A, lane 6). The time-course for elimination of the BoNT/E-altered SNAP-25 was similar following sequential toxin addition (illustrated here) as it was after exposure to BoNT/E alone (Fig. 2).

In parallel with the disappearance of the BoNT/E-truncated SNAP-25, intact and BoNT/A-altered SNAP-25 returned in preparations subjected to sequential serotype intoxication. Significantly, this occurred with no re-exposure to BoNT/A (Fig. 4A, lanes 3–6). During this period, both normal and BoNT/A-truncated SNAP-25 were found to increase in intensity. The rate of recovery of intact SNAP-25 was initially more rapid than the return of BoNT/A-truncated SNAP-25, but by day 18, the distribution of these proteins resembled that encountered prior to addition of BoNT/E (Fig. 4A, lanes 1 and 6).

Fig. 4B shows the results of a similar experiment in which BoNT/E addition was delayed for 60 days following exposure to BoNT/A (Fig. 4B). The apparent rate of synthesis of intact SNAP-25 and degradation of BoNT/E-truncated SNAP-25

Table 1
SNAP-25 sequence

SNAP-25 [residues 167-206]	Approx. MW
H_2N...-MGNEIDTQNRQIDR↓IMEKADSNKTRIDEANQ↓RATKMLGSG-COOH	25 kDa
BoNT/A-truncated SNAP-25	
H_2N...-MGNEIDTQNRQIDRIMEKADSNKTRIDEANQ-COOH	24 kDa
BoNT/E-truncated SNAP-25	
H_2N...-MGNEIDTQNRQIDR-COOH	22 kDa

BoNT/E site ↓ BoNT/A site ↓

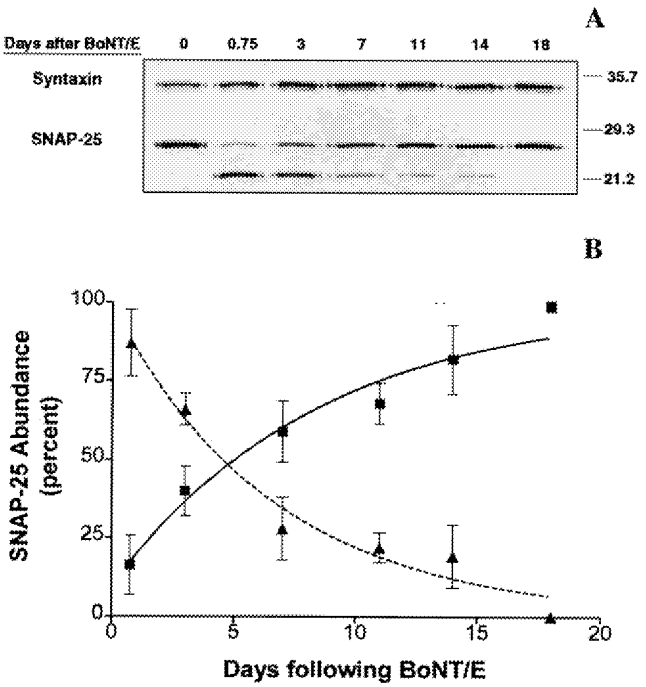

Fig. 2. Time-course for recovery of SNAP-25 following BoNT/E incubation. A: Exposure of spinal cord cell cultures to 116 pM BoNT/E for 18 h produced extensive cleavage of SNAP-25 (lane 2); 116 pM BoNT/E was the lowest concentration that produced near total loss of SNAP-25. To monitor recovery, toxin-containing medium was removed by aspiration and replaced with control growth medium; cultures were probed for SNAP-25 and syntaxin at 0.75, 3, 7, 11 and 18 days with anti-syntaxin and anti-SNAP-25 monoclonal antibodies; syntaxin remained unchanged while SNAP-25 levels increased progressively. B: Densitometric analysis of intact and BoNT/E-altered SNAP-25. The observed half-times for recovery of intact SNAP-25 (■) and elimination of BoNT/E-truncated SNAP-25 (▲) were 5.8 and 4.6 days, respectively. The values for synthesis were expressed relative to the band at zero time; values for degradation were expressed relative to the 22-kDa band at day 0.75.

were relatively unchanged from those observed 4 weeks earlier (Fig. 4A). Even with these later time points, reappearance and accumulation of BoNT/A-truncated SNAP-25 was observed as the abundance of new SNAP-25 increased (Fig. 4B, lanes 3–5). The relative proportion of BoNT/A-truncated to total SNAP-25, however, was only approximately half as much following 11 weeks of exposure to BoNT/A (Fig. 4B, lane 6) as it was after 6 weeks of exposure (Fig. 4A, lane 6).

The return of intact SNAP-25 indicates that active synthesis of this protein continues during the period of BoNT intoxication. The reappearance of BoNT/A-truncated SNAP-25 long after initial exposure to BoNT/A suggests that a fraction of newly synthesized SNAP-25 is cleaved by residual BoNT/A proteolytic activity. These findings are important, since they indicate that BoNT/A remains catalytically active in neurons with no observable decrease in activity for at least 43 days after initial exposure and only an approximate 2-fold decrease after 78 days.

4. Discussion

The clostridial neurotoxins, which include the seven serotypes of BoNT and tetanus toxin, are among the most potent substances known [1–3]. These toxins comprise a novel family of zinc metalloproteases with remarkable specificity for their target proteins [6]. Serotype A is further distinguished by having an exceptionally long duration of action [9,14], a key factor in its use for treatment of an increasing number of dysfunctions of muscle tone and movement [11]. The long duration of action of BoNT/A, however, renders management of systemic intoxication by this serotype especially difficult because individuals exposed to BoNT/A often require extended periods of artificial ventilation and intensive care [1–3]. The prolonged duration of BoNT/A action also creates problems arising from its potential military use, since pharmacological therapies would have to be maintained for the entire period of intoxication.

The basis for persistence of BoNT/A action is poorly understood. Two possibilities include (1) a continuous action of BoNT/A or of its catalytically active light chain in the nerve terminal, or (2) toxicity of one or both cleavage products of SNAP-25 [12,13]. An association between prolonged toxicity and continuous action of BoNT/A is readily apparent, although the mechanisms permitting prolonged survival of BoNT enzymatic activity in eukaryotic neurons would be novel.

Toxicity from the persistence of cleavage products is less obvious. Gutierrez et al. [12] observed that small 20–26-mer

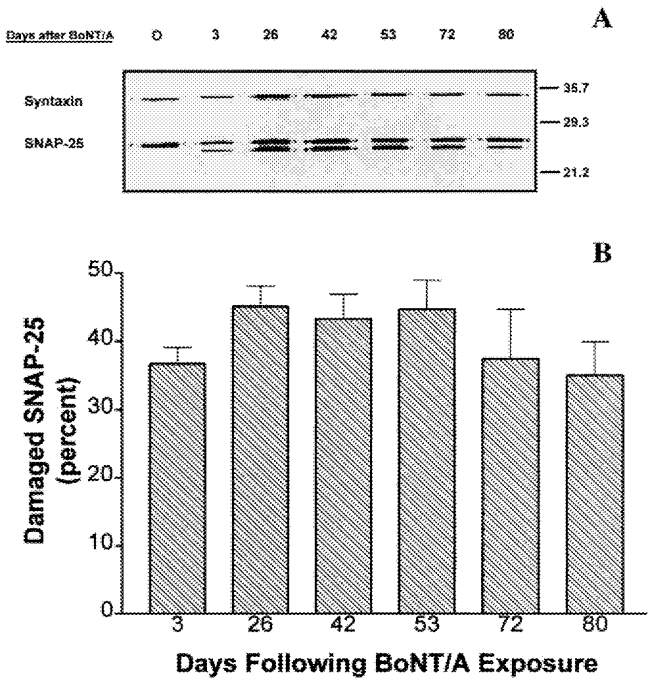

Fig. 3. A: Persistence of BoNT/A-truncated SNAP-25. Spinal cord cell cultures were treated with 0.4 pM BoNT/A for 48 h, which had been determined previously to cleave half of the SNAP-25 population. Excess toxin was removed by aspiration and replaced with growth medium; cultures were probed for SNAP-25 and syntaxin periodically during the next 80 days to monitor recovery. The levels of intact and BoNT/A-altered SNAP-25 were unaltered, suggesting an absence of recovery during the period of observation. B: Relative abundance of the 24-kDa SNAP-25 fragment produced by BoNT/A action over time. The percent of damaged SNAP-25 was calculated from the ratio of band intensities of BoNT/A-truncated SNAP-25 to total SNAP-25 at each time point. There is no statistically significant decrease in the amount of intact or damaged SNAP-25 over the 80-day period ($P < 0.05$). Each bar represents the average of band densities from three Western blots.

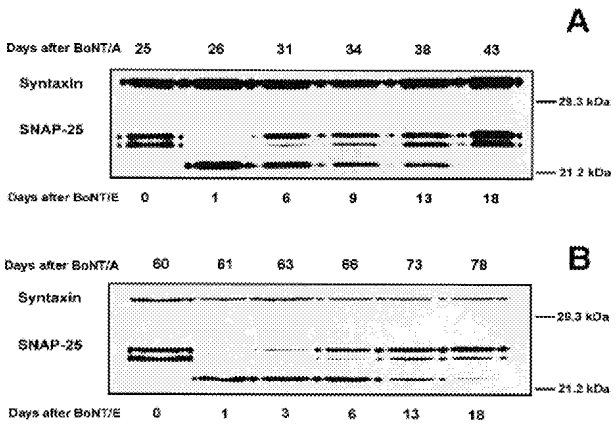

Fig. 4. Sequential additions of BoNT/A and E to demonstrate persistent action of BoNT/A. Cells were exposed to 0.4 pM BoNT/A for 72 h after which BoNT/A-containing medium was aspirated and replaced with control growth medium. Lane 1 represents 25 (A) or 60 (B) days after BoNT/A exposure. A: Cultures were exposed to 250 pM BoNT/E for 24 h, whereupon both intact and BoNT/A-truncated SNAP-25 were converted into the BoNT/E-altered form (lane 2). During the ensuing 18 days, increases were observed in intact and BoNT/A-truncated SNAP-25, while the BoNT/E-truncated SNAP-25 gradually disappeared (lanes 3–6). The high concentration of BoNT/E ensured complete proteolysis of both intact and BoNT/A-altered SNAP-25; the low BoNT/A concentration ensured that the long recovery times did not result from overloading cells with BoNT/A. B: Similar experiment as in A. but BoNT/E was added for 24 h 60 days after exposure to BoNT/A (lane 2). In spite of the longer residence time of BoNT/A, synthesis of intact SNAP-25 and accumulation of BoNT/A-truncated SNAP-25 continued to be observed (lanes 3–6). In each case, the return of BoNT/A-altered SNAP-25 indicates persistent activity of intracellular BoNT/A.

SNAP-25 peptides were able to inhibit the release of noradrenalin from permeabilized chromaffin cells by up to 70%. This action has been suggested to arise from the ability of these peptides to form non-productive fusion complexes with components of the vesicle docking and release apparatus [12,13]. The 24 kDa cleavage product has also been shown to inhibit release: in this case insulin secretion from a neuroendocrine cell line, presumably by competing with normal SNAP-25 [27].

The two mechanisms are not mutually exclusive, since continuous proteolysis of SNAP-25 by BoNT/A may be required to produce sustained concentrations of cleavage products. It is unlikely that, in the absence of continuous BoNT enzymatic activity, SNAP-25 fragments can persist for the observed duration of intoxication (Fig. 3). Evidence that at least some cleavage products degrade rapidly was shown in Fig. 2, where the 22 kDa BoNT/E-truncated SNAP-25 disappeared with a half-time of 4.6 days. The actual degradation rate is likely to be more rapid since new 22 kDa cleavage products are expected to add to the existing pool until BoNT/E activity ceases.

In the case of BoNT/A, the continued presence of the 24 kDa fragment observed in Fig. 3 may arise by one of two possible mechanisms: (1) BoNT/A activity is brief but the 24 kDa fragment escapes degradation, or (2) BoNT/A is significantly more stable than BoNT/E and acts continuously to cleave new SNAP-25 for the 80-day period of observation (Fig. 4B). If the first hypothesis is correct, the apparent equal distribution of the 24 kDa fragment relative to intact SNAP-25 (Fig. 3) requires concomitant block of SNAP-25 synthesis and inhibition of its degradation. However, the results shown in Fig. 4 demonstrate that SNAP-25 synthesis continues in the presence of both serotypes, and that return of SNAP-25 is followed by the reappearance of the 24-kDa fragment. Furthermore, because the 24-kDa fragment does not accumulate with time (Fig. 3) but appears to reach a steady-state with synthesis of intact SNAP-25, the 24-kDa fragment must be degraded at a rate similar to the turnover rate of intact SNAP-25. It would thus be difficult to conceive of a mechanism that would maintain the BoNT/A cleavage products for a time sufficient to account for the observed 80-day period in the absence of its continuous production (Fig. 2). Based on the above, inhibition of transmitter release from accumulation of cleavage products can account for only a fraction of the inhibition that is observed in the presence of toxin; the remainder must arise from the loss of SNAP-25 and the consequent impairment of vesicle fusion at productive release sites.

To attempt to quantify the contribution of the cleavage fragments to toxicity, precise knowledge of turnover rates is needed to determine the intracellular lifetimes of these products. However, detailed information of the rates of synthesis or degradation of SNAP-25 or its cleavage fragments is not presently available. The data in Fig. 2, for example, represents five separate processes: normal SNAP-25 synthesis and degradation, production and degradation of the 22-kDa SNAP-25 fragment, and loss of BoNT/E activity. Because these events could not be measured independently in the present study, our interpretations are confined to upper limit estimates. The actual rate of SNAP-25 synthesis, for example, may be faster than the observed half-time of 5.8 days in the absence of residual BoNT/E activity. Likewise, the observed degradation of the 22-kDa fragment may be slower than the actual rate because of residual BoNT/E activity.

Independent data for SNAP-25 degradation in the rabbit optic nerve demonstrated a half-time for degradation of 3 to 5 days with only trace amounts of labeled SNAP-25 remaining 16 days later [28]. As synthesis and degradation of intact SNAP-25 reaches a point of mass balance, new SNAP-25 must be synthesized at the same rate as it is degraded. Interestingly, the observed rate of synthesis for new SNAP-25 and degradation of the BoNT/E-truncated SNAP-25 occur at similar rates within experimental error, implying that BoNT/E is completely inactivated within 24 h of exposure.

A recent report by Eloepra et al. [29] indicated that in human volunteers, co-injection of BoNT/A and E results in rapid recovery of muscle tension similar to injections with BoNT/E alone. The authors concluded that both serotypes had equally short lifetimes, but paralysis was due to very slow degradation and inhibitory action of the 24-kDa SNAP-25 fragment. A brief lifetime for BoNT/A is not supported by the results in Fig. 4, which clearly indicate that BoNT/A action is prolonged. The differences between the present results and those of Eloepra et al. [29] may be due to species differences in the degradation of SNAP-25 cleavage products. Alternatively, co-injection of both serotypes may selectively block BoNT/A from entering the nerve terminal.

The experiments in Fig. 4 provide the clearest demonstration to date for the persistence of active BoNT/A after introduction of a relatively low concentration by showing the re-

turn of BoNT/A-truncated cleavage product long after BoNT/A exposure (Fig. 4B). Furthermore, the ratio of damaged to normal SNAP-25 was maintained for at least 43 days after the onset of BoNT/A exposure (Fig. 4A) and decreased only slightly 78 days thereafter (Fig. 4B). Fig. 4 also shows that SNAP-25 synthesis continues in spite of the presence of both toxins. This direct demonstration of SNAP-25 synthesis indicates that degradation of the 24-kDa fragment must be relatively rapid (Fig. 2). If this were not the case, the 24-kDa cleavage product would become more abundant as synthesis of new SNAP-25 proceeded. Since the 24-kDa fragment did not continue to accumulate over time (Fig. 3), it suggests that the major factor limiting recovery is prolonged stability of BoNT/A rather than depressed synthesis of SNAP-25 or suppressed degradation of cleavage fragments.

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